

## **Supplemental Material**

### **Methods**

#### **Evaluation of the optimal viability assay**

One control was to assess nonspecific interactions between the Ag-nps and the assay dye without the use of cells (“nanoparticle control”). In this case, Ag-nps dosing solutions equal to those used for viability tests were placed into collagen-coated 96-well plates devoid of cells and incubated for 24 h. The plates were then centrifuged at 1000rpm for 5 min and the media was replaced with the assay solution and read according to normal assay protocol as described above. Any change in absorbance values was reported and compared to control (KGM-2 only, no Ag-nps). The second control was to assess the specific interactions between the Ag-nps and the metabolized dye (“nanoparticle/cell control”). In this case, two plates were involved: in one 96-well plate, Ag-nps dosing solutions equal to those used for viability tests were placed into collagen-coated wells devoid of cells; in the other 96-well plate, cells were plated and grown. After incubating each plate for 24 h, the cell-containing plate was assayed according to normal viability protocol and read as described above. Immediately after reading, the particle-containing plate was spun down, the medium removed, and the assay solution from each well of the cell-containing plate was transferred into the corresponding well of the particle-containing plate. After incubation for 3 h, the plate was read and the difference in absorbance (before and after Ag-nps) was recorded.

#### **MTT**

The cell culture medium was removed from each treatment well and 200µl of 0.5mg/ml MTT (Sigma-Aldrich, St. Louis, MO) in KGM-2 warmed to 37°C was added to Ag-nps and vehicle-treated wells. The plates were incubated for 3 h, the MTT medium was removed, and

HEKs rinsed with 200µl of Hank's balanced salt solution (HBSS) for 2 min. The HBSS was removed, 100µl of 70% isopropanol was added to each well, and the plates were agitated for 25 minutes to extract the dye from the cells. Absorbance, directly proportional to cell viability, was determined at 550 nm in a Multiskan RC plate reader (Labsystems, Helsinki, Finland) equipped with Ascent software (version 2.6). The absorbance values were normalized by the controls and expressed as percent viability.

#### **aB**

One hundred microliters of cell culture medium was removed from each well and 10µl of aB (Molecular Probes, Invitrogen, Eugene, OR) solution was added to the medium. The plates were incubated for 3 hours, the fluorescence was quantitated (top read) on a Spectra Max Gemini EM spectrophotometer with an excitation wavelength of 545 nm and an emission wavelength of 590 nm. The fluorescence values were normalized by the controls and expressed as percent viability.

#### **96 AQ**

One hundred microliters of cell culture medium was removed from each well and 20µl of 96 AQ (Promega, Madison, WI) was added to the medium. The plates were incubated for 3 hours and then read at 450nm in a Multiskan RC microplate reader (Lab Systems, Helsinki, Finland) equipped with Ascent software (version 2.6). The absorbance values were normalized by the controls and expressed as percent viability.

#### **Cytokine Release**

The medium from each treatment set of the dosed cells was removed, pooled into a microfuge tube, and quickly frozen to -80°C until assayed. On the day of the assay, samples were thawed and 50µl of each sample added (in triplicate) to a filter plate with beads coupled to

the cytokine antibodies (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, Hercules, CA), and the assay was performed according to the manufacturer's instructions. Samples were assayed on a Bio-Plex System (Luminex x MAP Technology) equipped with Bio-Plex software (version 4.0). The samples were quantified by linear regression to logarithmic standard curves.

### **In vivo porcine skin exposure**

A 34µg/ml stock solution of each sample was prepared in deionized water and serially diluted (1:10) to provide Ag-nps solutions with concentrations ranging from 34.0 to 0.34µg/ml. Two female weanling pigs (*Sus scrofa*) weighing 20-30kg were housed in an AALAC accredited facility on elevated floors and were provided water and 15% protein pig and sow pellets ad libitum. The procurement, care, and use of animals were in accordance with the regulations and terms of the federal Animal Welfare Act and North Carolina State University's Institutional Animal Care and Use Committee guidelines. Approximately 24 h before the topical application of the Ag-nps, the pigs were sedated with an injection of telazol-ketamine-xylazine (TKX) and the excess hair on the dorsum carefully clipped. On the first day of the experiment each pig was sedated with TKX and placed in a sling. Fourteen sites, 7 on each side of the midline, were randomly assigned on the back of each pig. All sites were topically dosed with 500µl of the appropriate Ag-nps solution or control, allowed to air dry and then occluded with a Hilltop<sup>®</sup> chamber inset with a cotton pad (19mm inside diameter, 284mm<sup>2</sup> surface area; Hill Top Research Inc., Miamiville, OH, USA). The chambers were secured with non-irritating Medipore<sup>®</sup> (3M, St. Paul, MN) tape. Lastly, a body stocking was placed over the entire dorsum of each pig to further secure the chambers. Any change in the skin was recorded and photographed. After this, the pigs were redosed, the chambers were replaced and secured as described above and the dosing regiment was repeated for 14 days. At the conclusion of the 14

day study, a final Draize score was taken, all dosing sites were photographed, and the pigs were euthanized with 100mg/kg Euthasol<sup>®</sup> (Delmarva Laboratories, Inc., Midlothian, VA). The dosing sites were biopsied and placed in 10% NBF for light microscopy (LM) and in Trump's fixative for TEM. In addition, samples were also frozen in liquid nitrogen and stored at -80°C.

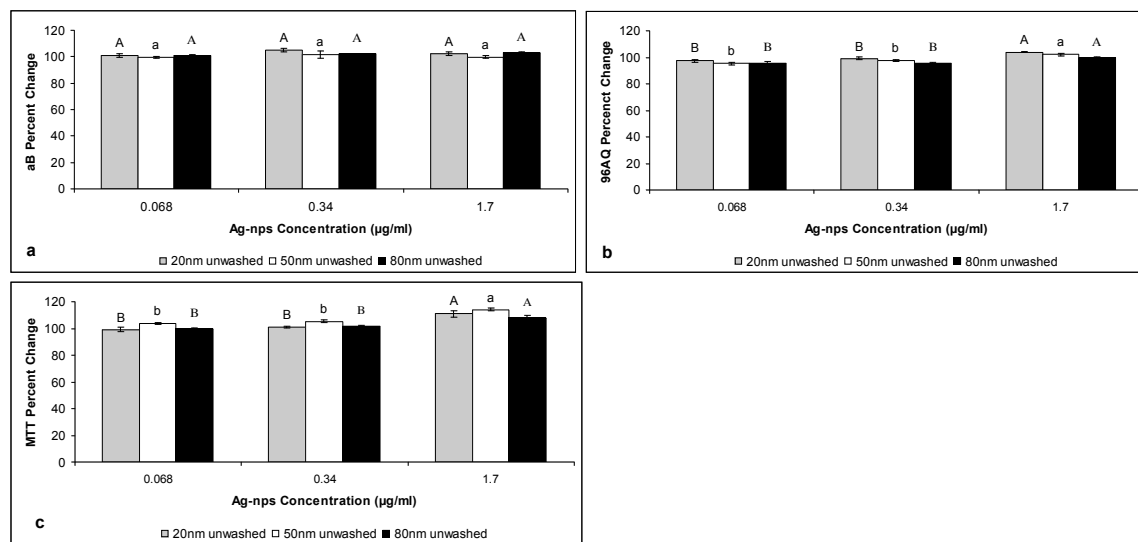
### **Ultrastructural Observations**

Samples were prepared by either diluting the washed and unwashed Ag-nps with deionized water, or suspending the carbon-coated Ag-nps in deionized water and sonicating for 10 minutes. Primary diameter was then determined using a Zetasizer (Malvern Instruments Ltd.) at 25°C. Additionally, to visualize particle morphology and uniformity of size, the samples were prepared by placing a drop of homogenous suspension of each Ag-nps (colloid; dry Ag-nps suspended in deionized water) onto a formvar-coated copper mesh grid and allowed to air dry. The samples were observed with an FEI/Philips EM 208S transmission electron microscope operating at an accelerating voltage of 80 kV.

HEK treated with Ag-nps were harvested with trypsin, rinsed in HBSS, and fixed for at least 24 h in Trump's fixative at 4°C. The cells were rinsed in 0.1M phosphate buffer (pH 7.2), pelleted in a microfuge tube, resuspended, and quickly pelleted in 3% molten agar. Agar-embedded samples were post fixed in 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) in 0.1M phosphate buffer for one hour at 4°C, washed with deionized water, dehydrated through an ascending ethanol concentration, cleared in acetone, infiltrated and embedded with Spurr resin, and polymerized at 70°C overnight. Cells were not stained to allow for better visualization of the Ag-nps and to ensure the absence of stain artifacts resulting from lead citrate and uranyl acetate.

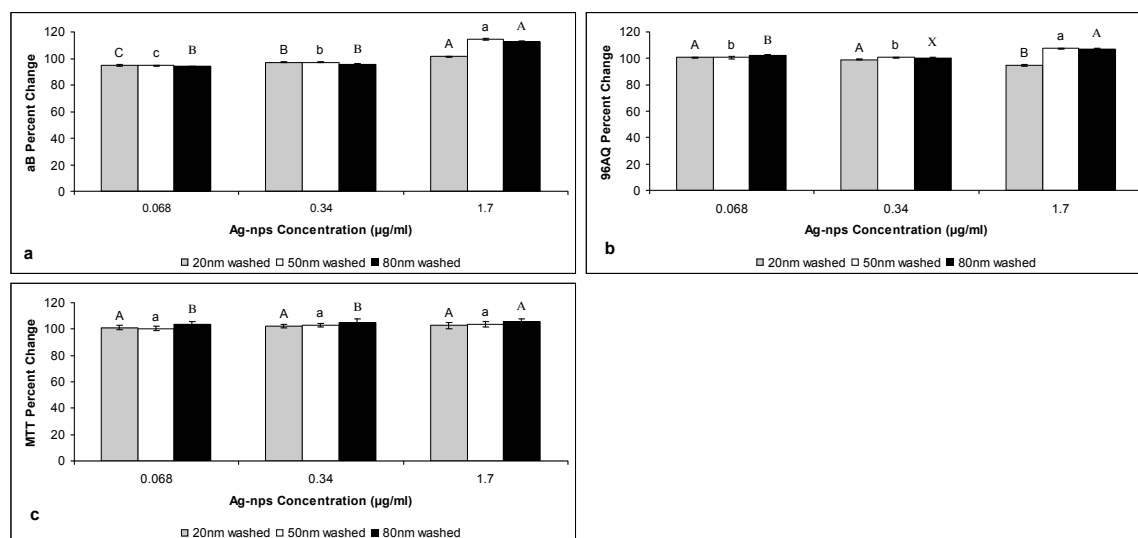
Skin treated with Ag-nps was harvested, trimmed into approximately 1mm<sup>2</sup> pieces and immersed in Trump's fixative at 4°C. The sections were then dehydrated and embedded in Spurr resin as described above. The samples were sectioned on a diamond knife and placed onto formvar-coated copper mesh grids for added stability and to help prevent both rolling of the edges and separation of the stratum corneum from the epidermis.

## Results



Supplemental Material, Figure 1: Nanoparticle control of Ag-nps after exposure to 20nm unwashed, 50nm unwashed, and 80nm unwashed Ag-nps. (A) aB; (B) 96AQ; (C) MTT.

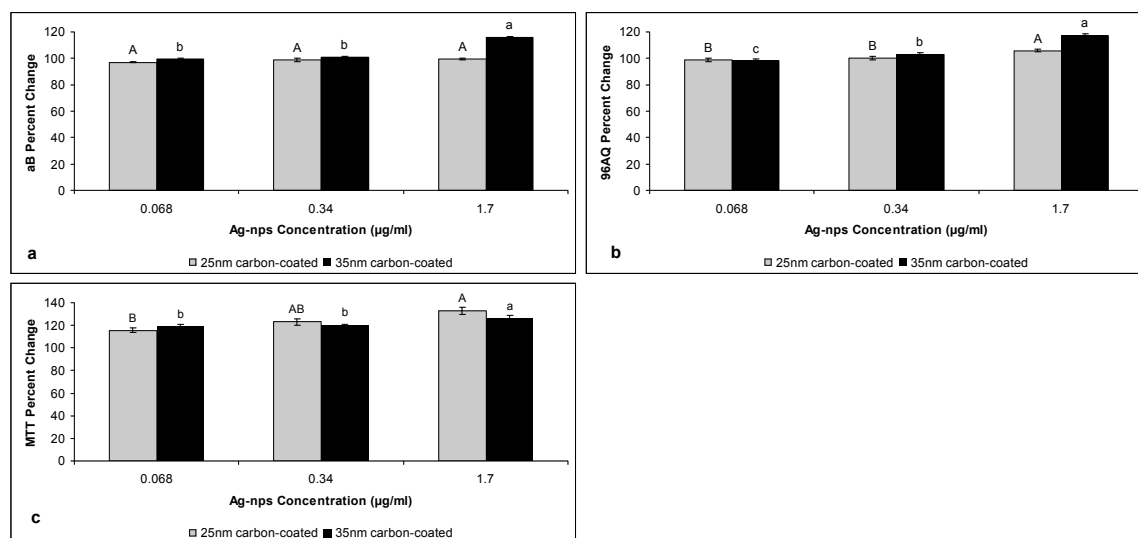
<sup>A</sup>p<0.05, 20nm unwashed; <sup>a</sup>p<0.05, 50nm unwashed; <sup>A</sup>p<0.05, 80nm unwashed, multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.



Supplemental Material, Figure 2: Nanoparticle control of Ag-nps after exposure to 20nm

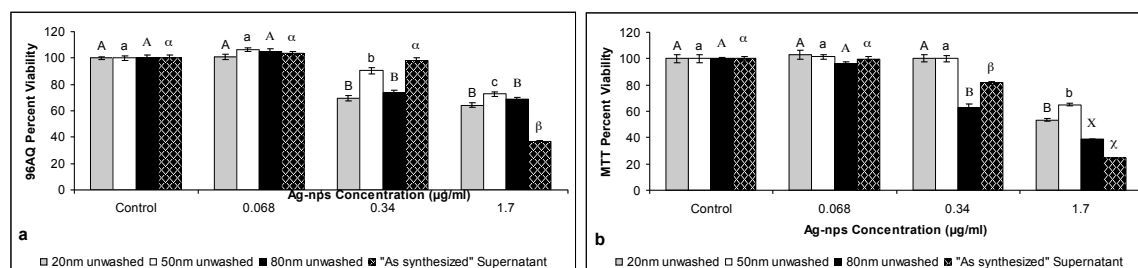
washed, 50nm washed and 80nm washed. (A) aB; (B) 96AQ; (C) MTT.

<sup>A</sup>p<0.05, 20nm washed; <sup>a</sup>p<0.05, 50nm washed; <sup>A</sup>p<0.05, 80nm washed, multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.



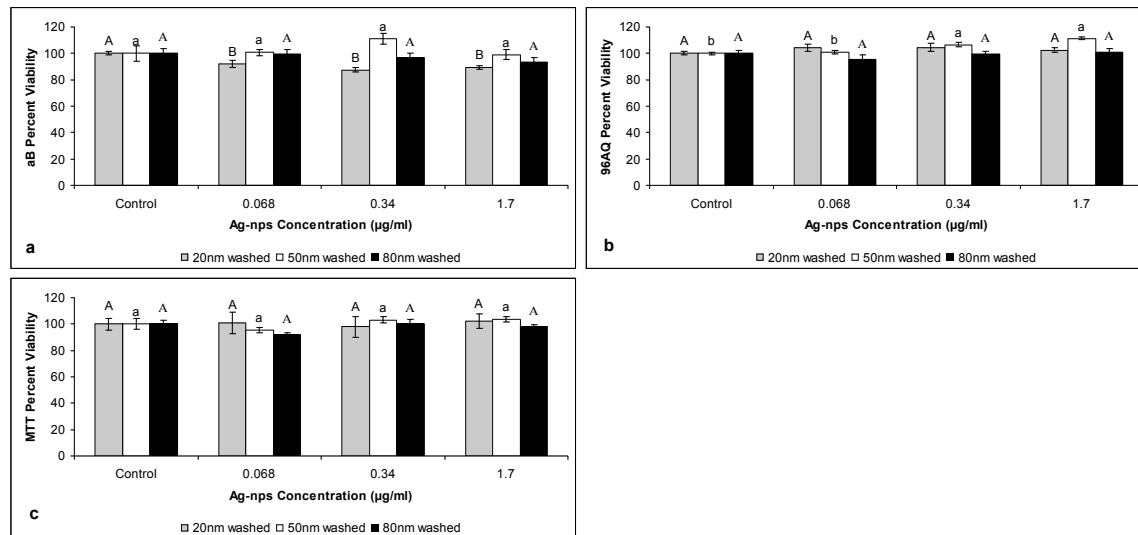
Supplemental Material, Figure 3: Nanoparticle control of HEK after exposure to 25nm carbon-coated and 35nm carbon-coated. (A) aB; (B) 96AQ; (C) MTT.

<sup>A</sup>p<0.05, 25nm carbon-coated; <sup>a</sup>p<0.05, 35nm carbon-coated; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.



Supplemental Material, Figure 4: Viability of HEK after exposure to 20nm unwashed, 50nm unwashed, and 80nm unwashed Ag-nps and "as synthesized" supernatant. (A) 96AQ; (B) MTT.

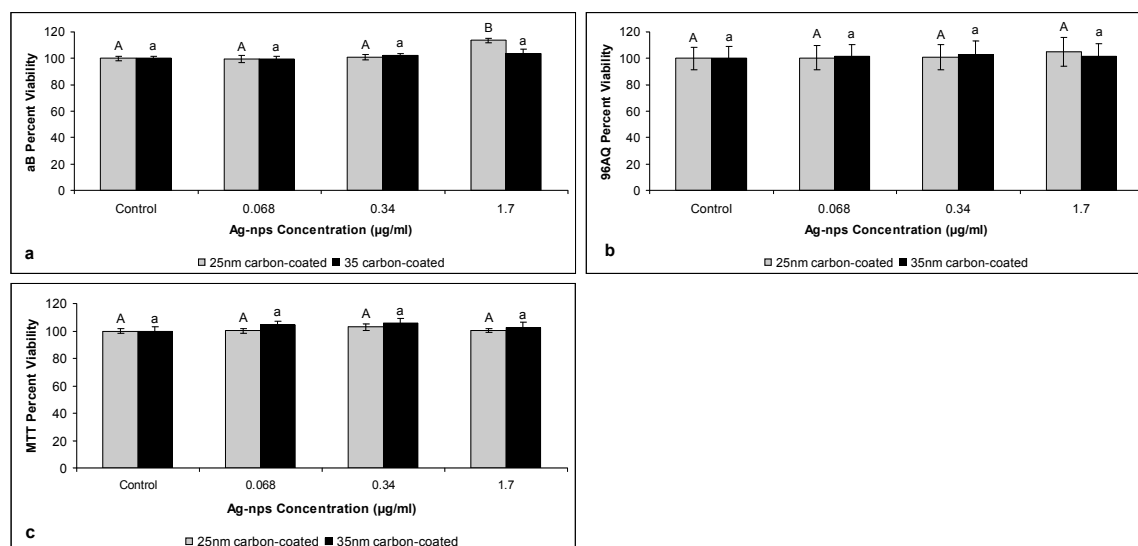
<sup>A</sup>p<0.05, 20nm unwashed; <sup>a</sup>p<0.05, 50nm unwashed; <sup>A</sup>p<0.05, 80nm unwashed; <sup>α</sup>p<0.05, "As synthesized" supernatant; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.



Supplemental Material, Figure 5: Viability of HEK after exposure to 20nm washed, 50nm washed and 80nm washed. (A) aB; (B) 96AQ; (C) MTT.

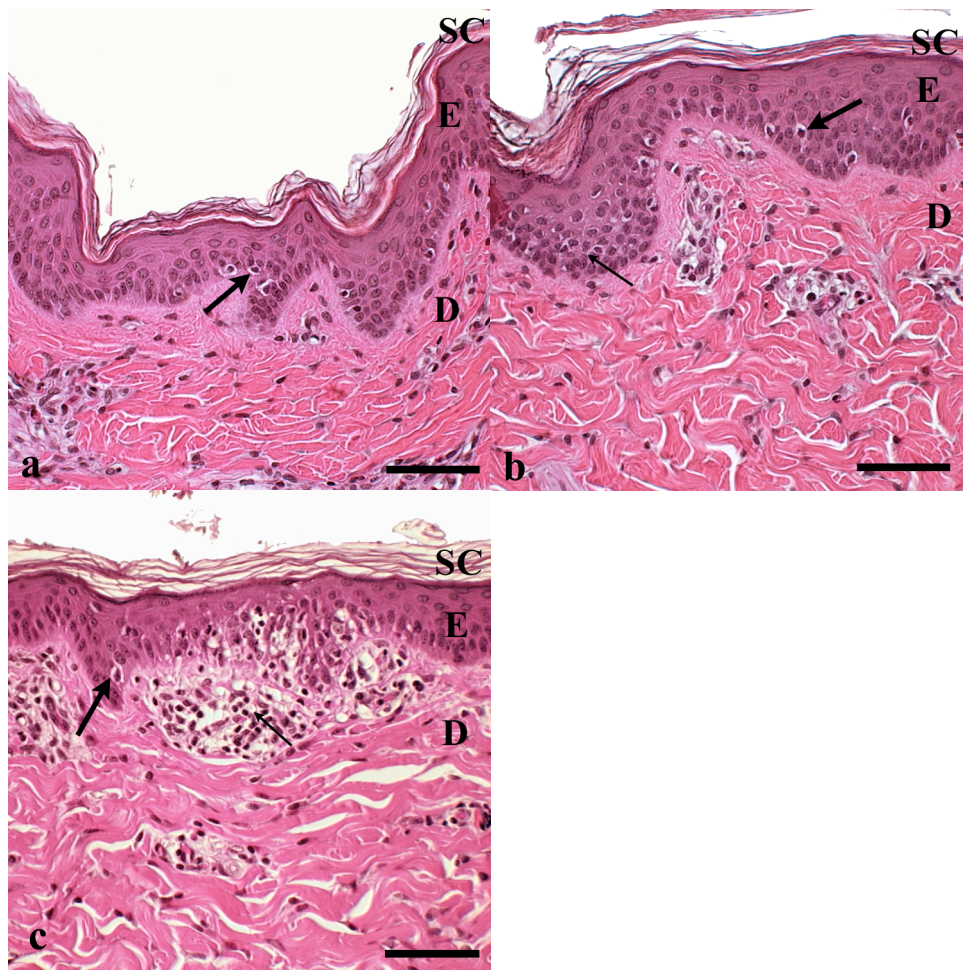
<sup>A</sup>p<0.05, 20nm washed; <sup>a</sup>p<0.05, 50nm washed; <sup>Δ</sup>p<0.05, 80nm washed; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.





Supplemental Material, Figure 6: Viability of HEK after exposure to 25nm carbon-coated and 35nm carbon-coated Ag-nps. (A) aB; (B) 96AQ; (C) MTT.

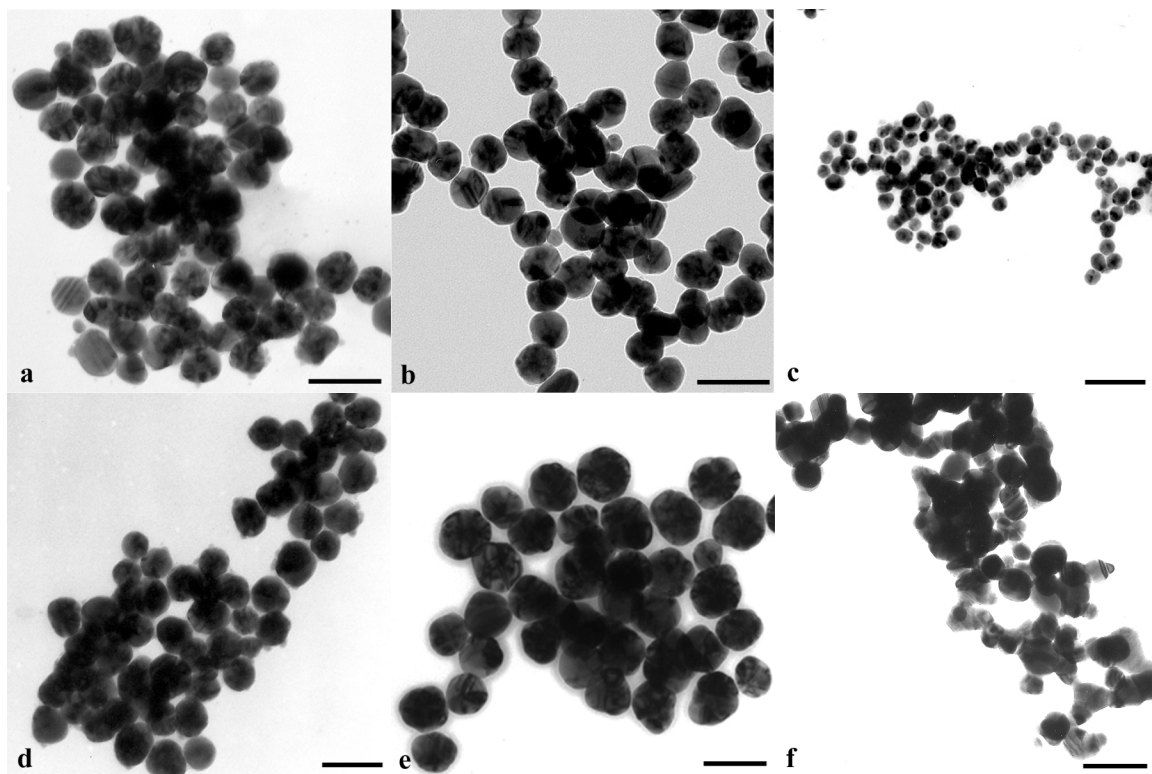
<sup>A</sup>p<0.05, 25nm carbon-coated; <sup>a</sup>p<0.05, 35nm carbon-coated; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.



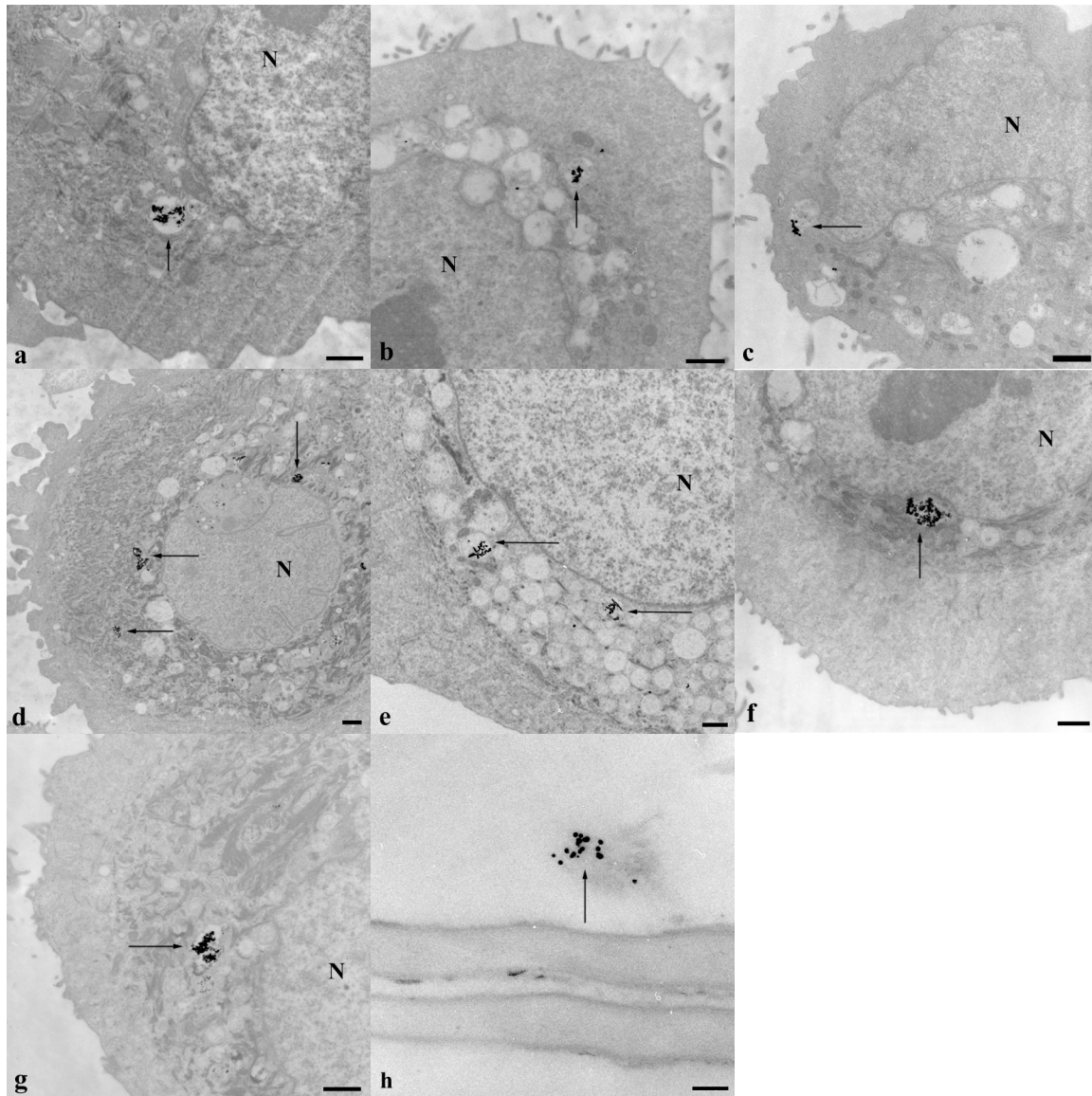
Supplemental Material, Figure 7: Light micrographs of skin treated with Ag-nps. (A) 20nm unwashed, 0.34μg/ml; (B) 20nm unwashed, 3.4μg/ml; (C) 20nm unwashed, 34μg/ml. Bars=60μm.

Large arrows point to intracellular epidermal edema, small arrows point to focal areas of intercellular epidermal edema.

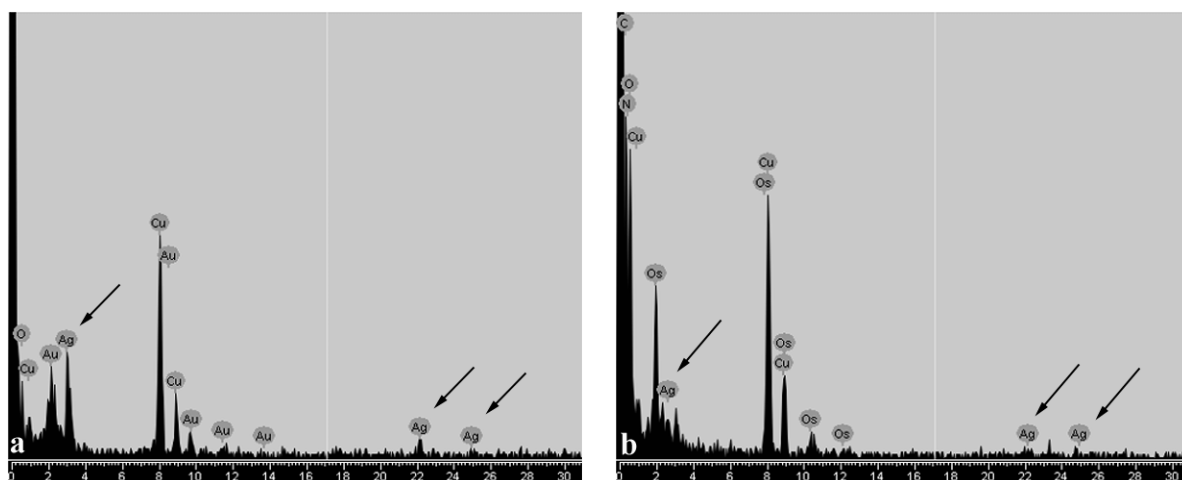
SC, stratum corneum; E, epidermis; D, dermis. H&E.



Supplemental Material, Figure 8: Transmission electron micrographs of Ag-nps. (A) 50nm unwashed; (B) 80nm unwashed; (C) 20nm washed; (D) 50nm washed; (E) 80nm washed; (F) 35nm carbon-coated. Bars=100nm.



Supplemental Material, Figure 9: Transmission electron micrographs of HEK and porcine skin exposed to different Ag-nps. (A) 20nm unwashed; (B) 50nm unwashed; (C) 80nm unwashed; (D) 20nm washed; (E) 50nm washed; (F) 25nm carbon-coated; (G) 35nm carbon-coated; (H) porcine skin and 34μg/ml of 20nm washed. HEK bars=1μm; Porcine skin bar=100nm. Arrows point to Ag-nps. N, nucleus; unstained sections.



Supplemental Material, Figure 10: Energy dispersive X-ray spectrum for samples dosed with Ag-nps (A) HEK dosed with 20nm washed; (B) porcine skin dosed with 20nm washed. Arrows point to Ag peaks. Au from Ag-nps core; Copper from grid; Osmium from tissue post fixation.